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INTRACELLULAR pH DECREASES DURING THE *IN VITRO* INDUCTION OF THE ACROSOME REACTION IN THE SPERM OF *SICYONIA INGENTIS*

FRED J. GRIFFIN, WALLIS H. CLARK JR., JOHN H. CROWE, AND LOIS M. CROWE

Department of Zoology, University of California, Davis, California 95616 and Bodega Marine Laboratory, Bodega Bay, California 94923

Abstract

Activation of the sperm of many invertebrate and some vertebrate species to undergo an acrosome reaction is accompanied by an increase in intracellular pH (pH_i). In each of these instances the pH_i of the unactivated cell is relatively low (6.9–7.4). Unactivated sperm of the marine shrimp, *Sicyonia ingentis*, possess an elevated pH_i (8.5). Induction of the acrosome reaction (exocytosis of the acrosomal vesicle and generation of an acrosomal filament) is accompanied by a decrease in pH_i (7.8). Low external pH elicits acrosomal filament formation in sperm that have undergone acrosomal exocytosis, but does not induce exocytosis in unreacted sperm. The ionophore, nigericin, enhances the percent of sperm that form filaments in low pH seawater (pH < 8.0), but does not elicit filament formation at external pHs ≥ 8.0. Valinomycin induces filament formation in sperm that have undergone exocytosis over a wide range of external pHs (5.75–8.5). The ability of valinomycin to induce filament formation in the upper portion of this pH range (8.0) declines as the extracellular K^+ concentration rises. These results demonstrate that the sperm of *S. ingentis* undergo a pH_i decrease as a result of the acrosome reaction and that the decrease is associated with acrosomal filament formation. In addition, they also suggest that an efflux of K^+ ions is connected to the pH_i decrease.

Introduction

As a prerequisite to fertilization, most sperm must first undergo acrosomal alterations, termed the acrosome reaction (Dan, 1952). Among the majority of invertebrate sperm and sperm of a few select vertebrates, the acrosome reaction (AR) is composed of the exocytosis of the acrosomal vesicle and generation of an acrosomal filament (reviewed by Dan, 1967; Austin, 1968). The AR occurs when a sperm contacts an egg-derived inducer. The inducer, a component of one of the egg investments, interacts with the plasma membrane of the sperm and initiates a series of ionic and biochemical sperm-associated events that lead to, among other sperm-associated changes, the AR (see Shapiro and Eddy, 1980; Lopo, 1983 for reviews). For example, the ionic changes associated with the AR in the sperm of *Strongylocentrotus purpuratus* include: (1) an uptake of extracellular Ca^{++} which is thought to be involved in the exocytosis of the acrosomal vesicle (Tilney *et al.*, 1978; Shackman *et al.*, 1981); (2) a Na^+ associated H^+ efflux which is necessary for the polymerization of actin filaments

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Abbreviations: ASW (artificial sea water), DMO (^{14}C-dimethyloxazolidine 2,4-dione), EW (egg water), pH_i (intracellular pH), pH_0 (extracellular pH).

Reprint requests to: W. H. Clark, P.O. Box 247, Bodega Bay, CA 94923.

and thus, the formation of the acrosomal filament; and (3) a K^+ efflux that leads to a depolarization of the sperm membrane potential (Shackman *et al.*, 1981). Although not thoroughly documented, it appears that the ARs of many motile invertebrate sperm involve the same ionic changes.

Unlike the sperm of most invertebrates, the sperm of the natantian decapod, *Sicyonia ingentis,* are nonmotile. These cells possess an anterior spike (contained within an acrosomal vesicle), a subacrosome, and a posteriorly located main body which houses the nucleus (Kleve *et al.*, 1980; Shigekawa and Clark, 1986). *S. ingentis* sperm do not possess flagella and also lack organized mitochondria. Sperm are transferred to female seminal receptacles during mating and stored until spawning, which may occur several weeks to months later (Anderson *et al.*, 1985). Thus, these sperm remain in an unactivated state for extended periods after transfer from the male. At spawning, ova are released from paired ovopores and mixed with sperm ejected from the seminal receptacles. Sperm bind spike first to ova and become activated to undergo a biphasic AR (Clark *et al.*, 1984). Within seconds bound sperm undergo the first phase of the AR, acrosomal exocytosis (which includes the loss of the spike), and 10–20 minutes later they complete the AR by generating an acrosomal filament (second phase). Thus, *in vivo* the two phases of the AR are temporally separated. Previous work demonstrates that the first phase, acrosomal exocytosis, depends upon external Ca^{++} (Clark *et al.*, 1981) as is true in other systems. These experiments were conducted with sperm taken from males, which are not competent to form acrosomal filaments (Clark *et al.*, 1984). The ionic requirements for acrosomal filament formation have not been investigated.

The ability to induce a complete AR in sperm removed from female seminal receptacles and incubated in isolated egg products enabled us to investigate the ionic requirements of the AR's second phase, acrosomal filament formation. The present paper: (1) describes the *in vitro* induction of a complete AR using egg components; (2) presents data suggesting that the sperm possess a high intracellular pH (pH_i) prior to undergoing the AR and that a pH_i decrease is associated with the second phase of the AR (formation of the acrosomal filament); and (3) provides data indicating that the outward movement of K^+ ions is involved in the pH_i drop.

Materials and Methods

Collection and maintenance of animals

Specimens of *Sicyonia ingentis* were collected using an otter-trawl in 60–100 meters of water off San Pedro, California. Live animals were transported in chilled seawater (8–10°C) to the Bodega Marine Laboratory and maintained in flow-through seawater tanks at ambient temperatures (10–14°C). Gravid females were isolated and kept under constant light in a 500 gallon flow-through tank. The lights were turned off to initiate spawning. Animals were monitored for spawning under a red light (Kodak Wratten #2 filter).

Collection of egg water

Spawning animals were removed from the tank and held over 50 ml glass beakers containing chilled (4°C) artificial seawater (ASW) prepared according to Cavanaugh (1956). After the negatively buoyant ova ($1–2.5 \times 10^5$) had settled to the bottom of the beaker, approximately ¾ of the seawater was drawn off. The ova were then resuspended by swirling and kept in suspension for five minutes. The remaining ASW

containing egg-derived components was then pipeted out of the beakers, cleared by centrifugation (100,000 × *g*, 15 min), and divided into 1 ml aliquots. The protein concentration of each egg water (EW) batch was determined after the method of Lowry *et al.* (1951). EW was stored in liquid nitrogen if not used immediately.

Collection of sperm

In *S. ingentis*, only sperm that have been transferred to a female and stored in the female's seminal receptacles are competent to: (1) undergo the acrosome reaction (AR) in response to egg derived components; and (2) form an acrosomal filament as part of the AR, regardless of the manner of induction (Clark *et al.*, 1984). As a result, only sperm taken from seminal receptacles of the female were used. Seminal receptacles from ten or more females were pooled, homogenized in ASW using a Wheaton 5 ml tissue grinder to free sperm, and hand centrifuged to remove fragments of empty receptacles. Free sperm were pelleted from the supernatant at 200 × *g* for five minutes. Pelleted sperm were resuspended in ASW and used within one hour of isolation.

Induction of the acrosome reaction with egg water

Isolated sperm (10^6 cells) were incubated in 1 ml of experimental (containing EW) and control (containing ASW) solutions. Aliquots of cells in each experiment were fixed (with a drop of 5% glutaraldehyde in ASW) at appropriate times and scored with phase microscopy (400×) for: (1) percent unreacted; (2) percent that had undergone acrosomal exocytosis but had not formed acrosomal filaments; and (3) percent fully reacted (sperm which possessed acrosomal filaments). For each experimental run (n), duplicate 20 μl aliquots were removed and 100 sperm were scored in each for acrosomal status.

Intracellular pH determinations

Isolated sperm (2.1×10^8) were divided into three equal samples. One sample was incubated for 10 min in 1.6 ml of ASW (pH 8.0); this sample was used to measure the pH_i of unreacted sperm. A second sample was incubated in 1.6 ml of EW (pH 7.8) for 10 min; these sperm were used to measure the pH_i of sperm that had undergone acrosomal exocytosis. The last sample was incubated in 1.6 ml of EW for 50 min; these sperm were used to measure the pH_i of fully reacted sperm. Intracellular pH determinations were made with ^{14}C-dimethyloxazolidine 2,4-dione (DMO) using a modification of the technique described by Waddell and Butler (1959). At the conclusion of the initial incubations, each of the three samples was divided in half: (1) 25 μl of ^3H_2O and 50 μl of ^{14}C inulin were added to one; and (2) to the other, 25 μl of ^3H_2O inulin and 50 μl of ^{14}C DMO (final concentration of 33 μ*M*) were added. After a 20 min equilibration period: (1) triplicate 20 μl aliquots (controls) were transferred to scintillation vials containing 15 ml of ACS scintillation fluid (Beckman); (2) 10 μl samples were removed, fixed, and scored for acrosomal status; and (3) triplicate 200 μl samples were microfuged (Fisher Model #235B) for 90 seconds through a 95 volume percent silicone oil (Dow Corning 704)–5 volume percent hexane solution, the supernatants were removed, and the tips of the microfuge tubes (containing the sperm pellets) were cut off and placed in scintillation fluid. The samples sat overnight and were then counted on a Beckman LS100 scintillation counter. Calculation of internal water space and pH_i determinations followed those described by Shackman *et al.* (1981).

pH induction experiments

Sperm (10^7/ml) were incubated for 5 min in either ASW pH 8.0 or EW pH 8.0 after which 100 μl aliquots were added to 900 μl of ASW at the following pHs: 5.75, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, and 9.5. Reactions were halted 10 min later with a drop of 5% glutaraldehyde (in ASW) and acrosomal status was scored. In experiments using sperm that had spontaneously undergone acrosomal exocytosis in ASW alone, sperm were scored until 100 reacted cells had been observed in each duplicate. In those using EW to induce acrosomal exocytosis, counts were performed as described above. The pH of ASW was determined on an Orion (EA920) pH meter. Above pH 8.0, ASW was adjusted with 0.1–1.0 *N* NaOH; below pH 8.0 it was adjusted with 0.1–1.0 *N* HCl or 0.2 *M* acetate buffer. All ASWs were pH adjusted just prior to use.

Ionophore induction experiments

In separate experiments, sperm (10^7/ml) were induced to undergo acrosomal exocytosis in EW as described above and 100 μl aliquots were added to 900 μl of ASW at the pHs described in the previous section. Immediately after the addition of sperm, 5 μl of nigericin (0.5 m*M* in 100% DMSO) or valinomycin (1.0 m*M* in 100% DMSO) were added, with mixing, to the sperm suspensions. Control samples contained 0.5% DMSO. Aliquots of each treatment were fixed with a drop of 5% glutaraldehyde (in ASW) 5 minutes after the addition of the ionophores or DMSO and sperm were scored for acrosomal status. In addition, samples were removed and fixed to determine levels of acrosomal exocytosis prior to introduction into pH ASWs and the addition of the ionophores.

ASWs of different [K^+] were obtained by adding or deleting equal molar amounts of KCl and NaCl from the MBL formula for ASW (Cavanaugh, 1956).

Results

Induction of the acrosome reaction by egg water

Acrosomal status of *S. ingentis* sperm is easily scored with phase microscopy. Figure 1 illustrates an unreacted sperm, a sperm that has undergone acrosomal exocytosis, and a fully reacted sperm (possessing an acrosomal filament). Sperm removed from the seminal receptacles of females and incubated in 50 μg/ml (protein) of egg water (EW) undergo a complete AR (acrosomal exocytosis and formation of an acrosomal filament) in which the temporal separation between the two phases is maintained. Within 1 min of exposure to EW, *S. ingentis* sperm underwent acrosomal exocytosis at levels greater than ASW controls (41% as compared to 7.3%) and by 5 min, greater than 75% of the sperm had undergone acrosomal exocytosis (Fig. 2). These sperm (after a 15 min incubation in EW) had only undergone acrosomal exocytosis; they did not possess acrosomal filaments. Sperm that had undergone acrosomal exocytosis did begin to form filaments, commencing approximately 30 min after introduction into EW (Fig. 3). At 45 min, more than 50% of all sperm counted possessed acrosomal filaments; this translates into more than two-thirds of exocytosed sperm possessing filaments. By 60 min, approximately 60% of all sperm (85% of exocytosed sperm) had formed filaments. The sperm that formed acrosomal filaments were those that had undergone acrosomal exocytosis. The total percent of sperm that had either undergone only exocytosis or undergone a complete AR remained constant through 60 min (Fig. 3).

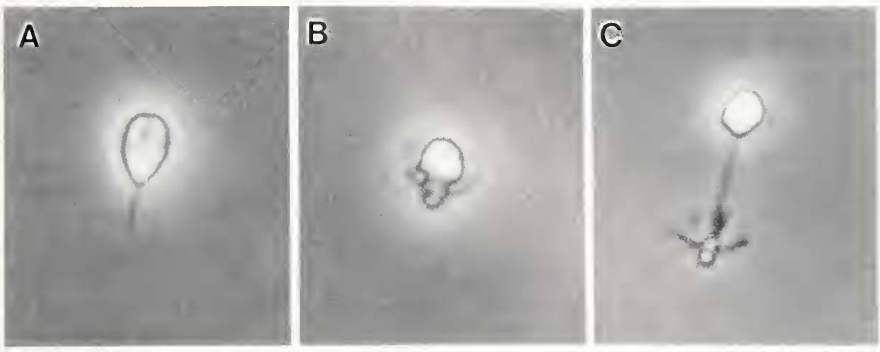

FIGURE 1. Phase micrographs of the three activational states of *Sicyonia ingentis* sperm; (A) an unreacted sperm possessing an anterior spike, (B) a sperm that has undergone acrosomal exocytosis and has lost the spike, and (C) a fully reacted sperm possessing an acrosomal filament.

A small but consistent number of sperm (8–10%) isolated in artificial seawater (ASW), and not transferred to EW, underwent acrosomal exocytosis (Fig. 2). This percent not only remained constant with increased incubation times, but exocytosis was the only portion of the AR that occurred. Sperm isolated to ASW have been observed for up to 180 min without seeing acrosomal filaments.

Intracellular pH measurements

Based on the accumulation ratios of the DMO uptake experiments we have calculated an average pH_i for unreacted and reacted *S. ingentis* sperm. Unreacted sperm

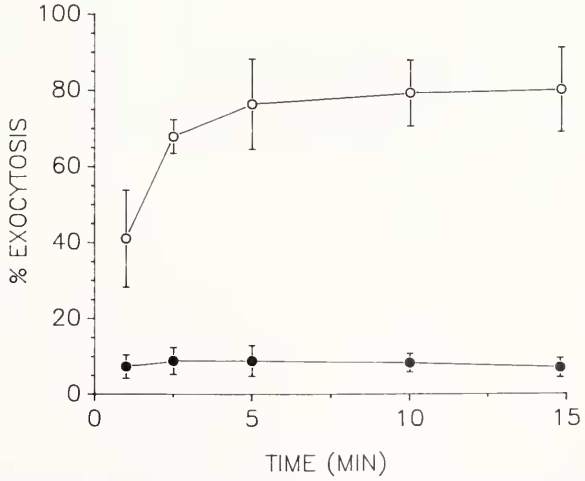

FIGURE 2. EW induction of acrosomal exocytosis; response over time. Sperm were incubated in 50 μg/ml EW (○) or ASW (●), fixed at the times designated above, and scored for acrosomal status. Data points are means; vertical lines are standard deviations (n = 4). Each replicate utilized a separate batch of sperm and EW.

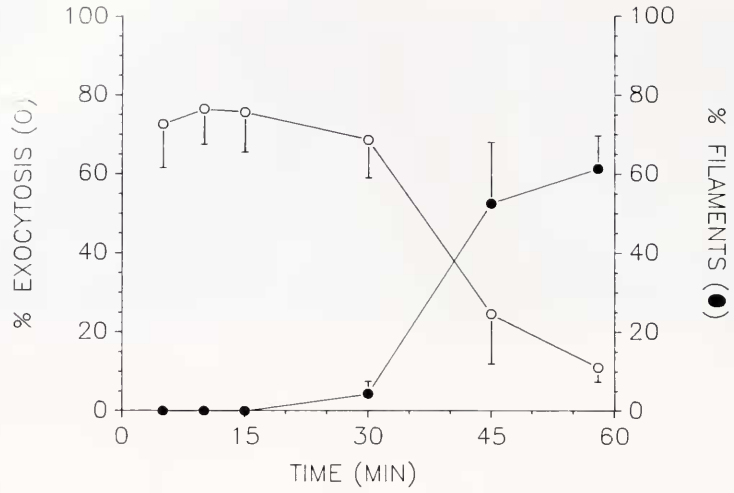

FIGURE 3. Acrosomal filament formation as a function of time. Sperm were incubated in 50 μg/ml of EW. Aliquots of sperm were removed, fixed, and scored for percent exocytosis (○) and percent formed filaments (●) at each time point. Data points are the means of four replicates; each replicate was conducted with different batches of sperm and EW. Vertical lines are standard deviations.

removed from seminal receptacles and placed in ASW (pH 8.0) possessed a pH_i of 8.47 ± 0.27. In these samples, greater than 91% of the sperm were unreacted and none of the reacted sperm possessed acrosomal filaments (Table I). Sperm incubated in EW (pH 7.8) for 10 min prior to the addition of the DMO possessed a significantly lower pH_i of 7.81 ± 0.13 ($P < 0.05$). Greater than 74% of these sperm had undergone acrosomal exocytosis and approximately 3% possessed acrosomal filaments at the end of the equilibration period (30 min after sperm had been introduced into EW). The pH_i of sperm incubated in EW for 50 min, 8.01 ± 0.06, was also lower than that of unreacted sperm ($P < 0.05$), but was not significantly different from the 10 min EW samples. Seventy-one percent of the sperm incubated in EW for 50 min possessed filaments at the end of the equilibration period (70 min after introduction into EW).

TABLE I

pH_i of Sicyonia ingentis *sperm*

Sample	Exocytosed[1]	Filaments[2]	pH_i
ASW	8.3 ± 2.5	0	8.47 ± 0.27
EW_{10}	72.3 ± 3.1	2.7 ± 1.5	7.81 ± 0.13
EW_{50}	6.0 ± 2.0	71.0 ± 2.0	8.01 ± 0.06

Isolated sperm were reacted in EW for 10 min (EW_{10}) and 50 min (EW_{50}) and used to measure pH_i of sperm that had undergone only acrosomal exocytosis and sperm that had fully reacted, respectively. The pH_i of unreacted sperm (ASW) was measured after incubating isolated sperm for 10 min in ASW.

[1] Percent of sperm which had undergone acrosomal exocytosis only at the time of disruption.

[2] Percent of sperm which had undergone a complete AR at the time of disruption.

Effect of external pH on the AR

External pH (pH_0), within the range examined, does not elicit the first phase of the AR, acrosomal exocytosis, in *S. ingentis* sperm (Fig. 4A). The percentages of sperm that were unreacted after transfer to the pH ASWs did not vary significantly with pH_0. This was true for those sperm that had been preincubated in ASW alone, as well as for those sperm that had been preincubated in EW. In those experiments where sperm had been preincubated in EW, the percent that did not undergo acrosomal exocytosis averaged 26.1 for all pH_0s with no observable pH-dependent trend. The same held true for those sperm that were not exposed to EW (incubated in ASW only); the percent of these sperm that did not react averaged 90.7 for all pH_0 treatments. Such was not the case with regard to acrosomal filament formation.

Exposure of sperm to low pH_0 did elicit the formation of acrosomal filaments. Sperm induced to undergo exocytosis with EW and subsequently transferred to pH_0s of less than 7 underwent filament formation within 10 min of transfer (Fig. 4B). This represents a reduction of the temporal separation between the two phases of 20–35 min. The pH_0 optimum for filament induction was between pH 5.75 and 6.5. Below pH 5.75, exocytosed sperm were disrupted and above pH 7.0, sperm did not form acrosomal filaments within the reduced temporal window. Similar results were obtained with sperm that had spontaneously undergone exocytosis in ASW (not incubated in EW). The percentages of these sperm that formed filaments after exposure to low pH_0 were somewhat less than the sperm treated with EW, but the effect of pH_0 was similar (Fig. 4).

Induction of acrosomal filament formation by ionophores

The response of sperm to low pH_0 was enhanced with the addition of either nigericin or valinomycin, however, the pH_0 optima were different for both ionophores (Fig. 5). Greater than 50% of sperm that had undergone acrosomal exocytosis in EW and were subsequently exposed to nigericin for 5 min underwent filament formation in pH_0 6.0–7.5. At pH_0 5.75, 31.5 ± 8.2% of such sperm possessed filaments, however, the number of unreacted sperm was twice that of the other pH_0 treatments, suggesting that at pH_0 5.75 reacted sperm were disrupting (Fig. 5A). With the addition of nigericin, not only were the percentages of filament formations increased at low pH_0s (6.0–7.5), but the range for filament induction was shifted 0.5–1.0 units basic.

Valinomycin not only elicited more filament formations than any of the other treatments, but it was also effective over a broader range of pH_0s than the other treatments (Fig. 5). Greater than 80% of exocytosed sperm underwent filament formation in pH_0 5.75–8.0. At pH_0s 9.0 and 9.5 the percentages of filaments were dramatically reduced. As in low pH_0 inductions in the absence of ionophores, there was no observable effect on acrosomal exocytosis (Fig. 5A).

The ability of the ionophore valinomycin to induce formation of acrosomal filaments was pH_0 dependent when extracellular K^+ was elevated (Fig. 6). Filament formation in pH 6.0 ASW was not reduced by increasing concentrations of extracellular K^+, however, a steady decline in the percentage of filaments was observed in pH 8.0 ASW as the K^+ level was increased. When the $[K^+]$ was increased from 10 m*M* to 20 m*M*, filament formation at pH_0 8.0 decreased by approximately 50%. At 30 m*M* K^+ filament formation declined by another 50%, and at 40 m*M* K^+ no filaments were observed. In pH 9.0 ASW, filaments were only formed in low K^+ (K^+-free ASW).

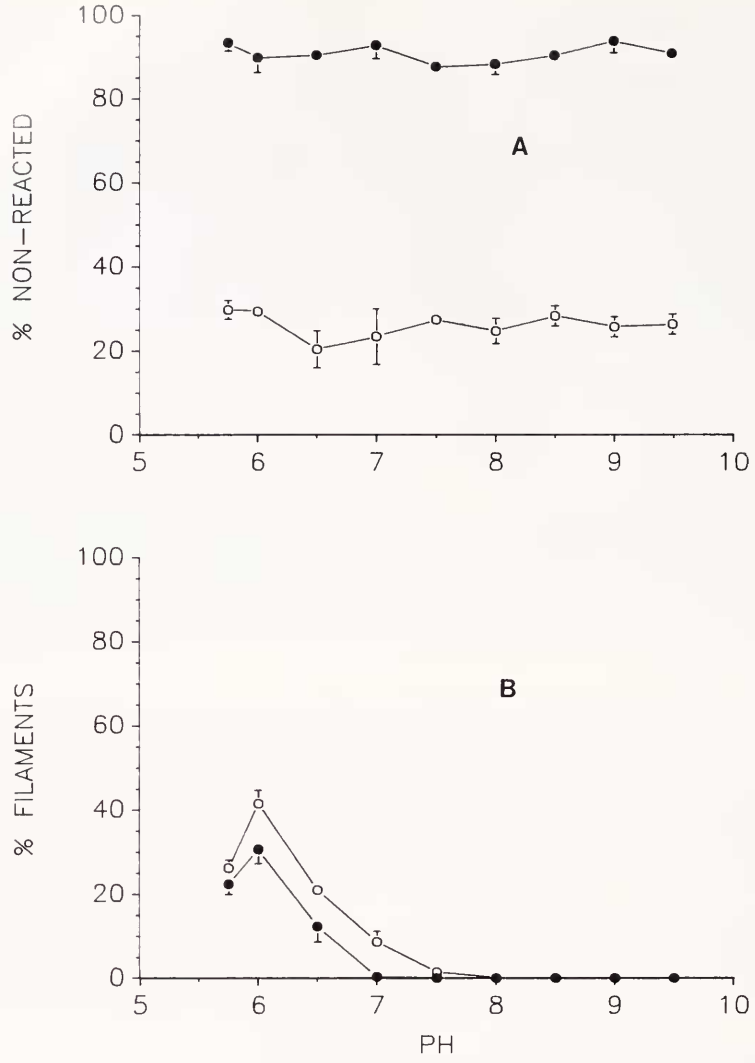

FIGURE 4. Effects of external pH upon the AR. (A) Acrosomal exocytosis. (B) Acrosomal filament formation. Sperm (10^7/ml) were incubated for 5 min in either ASW pH 8.0 (●) or EW pH 8.0 (○) after which 100 μl aliquots were added to 900 μl of ASW at the pHs indicated above. Reactions were halted 10 min later with a drop of 5% glutaraldehyde (in ASW) and acrosomal status was scored. Data points in (B) represent mean % of exocytosed sperm that formed filaments.

DISCUSSION

Induction of the two phases (acrosomal exocytosis and acrosomal filament formation) of the AR in *S. ingentis* sperm is temporally separated and sequential *in vivo* (Clark *et al.*, 1984). Upon binding to ova, sperm undergo acrosomal exocytosis and some 10–20 min later undergo acrosomal filament formation. The present report has demonstrated that the *in vitro* induction of this AR in sperm removed from female

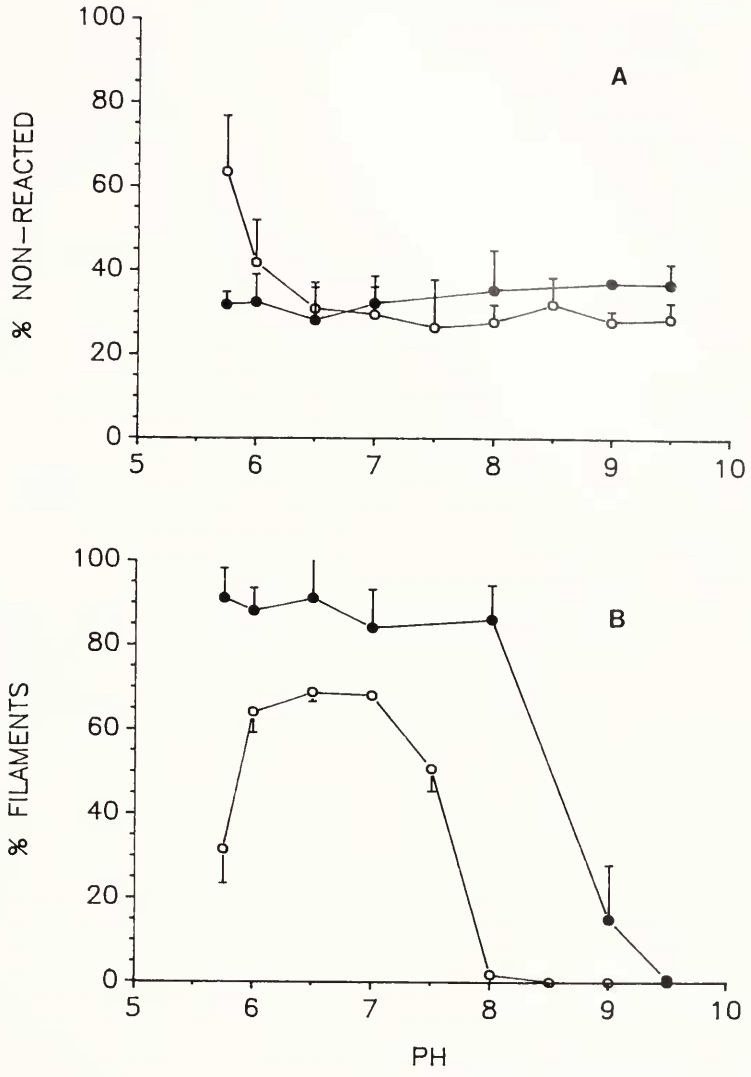

FIGURE 5. The effects of pH_0 on valinomycin and nigericin induction of the AR. (A) Acrosomal exocytosis. (B) Acrosomal filament formation. Sperm (10^7/ml) were first induced to undergo acrosomal exocytosis in EW, 100 μl aliquots were added to 900 μl of ASW at the pHs indicated above, and then exposed to either 1 μM nigericin (○) or 5 μM valinomycin (●). Sperm were fixed after 5 min and scored for reactions. Data points in (B) represent mean percent of exocytosed sperm that formed acrosomal filaments; vertical lines are standard deviations. n = 3. Each n in each experimental batch represents sperm pooled from different females, different ASWs, and different EW and nigericin solutions.

seminal receptacles and incubated in solutions containing isolated egg components (EW) is also temporally separated and sequential. *In vitro*, acrosomal exocytosis is achieved within 2.5–5 min, yet sperm that have undergone acrosomal exocytosis do not form acrosomal filaments for an additional 30–45 min. Thus the temporal separation that is observed on the surface of an ovum is preserved albeit lengthened under

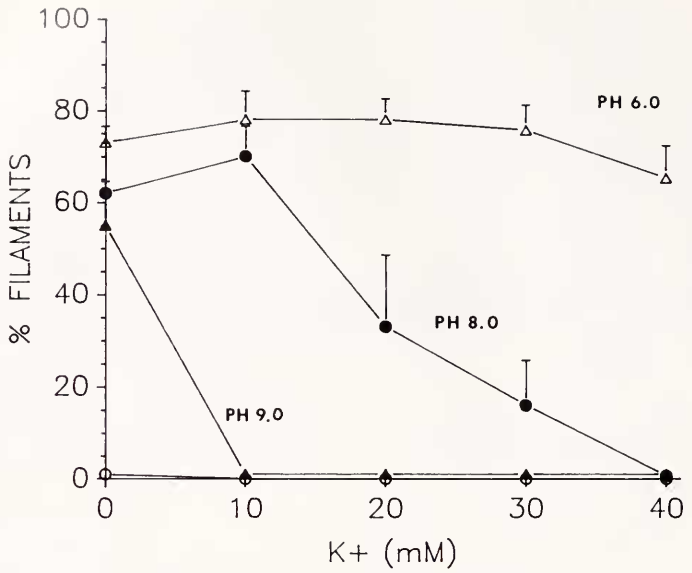

FIGURE 6. Acrosomal filament formation and external K^+. Sperm (10^7/ml) were induced to undergo acrosomal exocytosis in EW (5 min). One hundred (100) μl samples were then transferred to 900 μl of ASWs containing from 0 to 40 m*M* K^+ at pH_0 6.0 (△), pH_0 8.0 (●), and pH_0 9.0 (▲). Samples were fixed at 5 min and scored for acrosomal status. Control samples (○) were preincubated in ASW (not exposed to EW), added to ASW (pH 8.0) containing the described [K^+], and exposed to 0.5% DMSO. Data points are mean percent of exocytosed sperm that formed filaments (n = 3); vertical lines are standard deviations.

in vitro conditions. The ability to elicit a complete AR *in vitro* and the fact that the two phases are separated has allowed the dissection of the two phases with respect to the controls of activation. Based upon direct measurements of pH_i, low pH_0 inductions of the AR, and the effects of both low pH_0 and external [K^+] on ionophore inductions, we propose that formation of the acrosomal filament in *S. ingentis* sperm is associated with a pH_i decrease.

Measurements of intracellular pH in *S. ingentis* sperm suggest that: (1) unreacted sperm possess a high intracellular pH; (2) prior to formation of the acrosomal filament these cells undergo a pH_i decrease; and (3) subsequent to filament formation they do not return to the unactivated pH_i. Although DMO is a widely used probe for determining pH_i, it does have limitations (Roos and Boron, 1981; Busa and Nuccitelli, 1984). These include: (1) DMO measurements reflect an average pH for the cell and do not provide information on the pH of subcellular compartments (*e.g.*, the acrosomal vesicle or the subacrosome); and (2) alkaline membrane-bound organelles can sequester DMO, giving an erroneous picture of the pH of other subcellular compartments (Roos and Boron, 1981; Busa and Nuccitelli, 1984). For example, Grinstein *et al.* (1984) have reported that a DMO measured pH_i increase at lymphocyte proliferation is in fact not an activational pH_i change, but rather an increase in the number of mitochondria (which results in an increased DMO uptake by the cells). The structural organization and the direction of the measured pH_i change in *S. ingentis* sperm, however, allowed us to entertain the supposition that the pH_i change was real and was associated with filament formation. Unreacted *S. ingentis* sperm possess three subcellular regions: a nucleus, a subacrosome, and an acrosomal vesicle;

mature sperm do not possess mitochondria (Shigekawa and Clark, 1986). As a result, any pH_i changes would be expected to be associated with one of these compartments and two of them are involved in the AR. We would not expect, *a priori,* an overall pH_i decrease to occur simply as a result of acrosomal exocytosis; the acrosomal vesicle is an acidic organelle (Kleve *et al.,* 1980) and therefore its loss at exocytosis might be expected to yield an increase in average pH_i. It was therefore reasonable to expect the pH_i changes to be associated with the subacrosome.

Results of the low pH_0 induction experiments correlate well with the pH_i measurements and delineate at which phase of the AR the pH_i drop occurs. Neither low pH ($\leq$7.5) alone nor low pH in conjunction with nigericin or valinomycin induce unreacted sperm to undergo acrosomal exocytosis. All three do induce acrosomal filament formation in sperm that have undergone exocytosis. It follows that the pH_i decrease is associated with the second phase of the AR, formation of the acrosomal filament. Furthermore, low pH_0 elicits filament formation in sperm that have exocytosed in ASW and have not been exposed to EW. This indicates that the pH_0 is not acting through a pH alteration of EW, rather, it is directly influencing filament formation. These observations are in contrast to previous studies demonstrating that a net rise in pH_i occurs during the AR in sperm of other species (Shackman *et al.,* 1981; Working and Meizel, 1983; Matsui *et al.,*1986). By contrast with the sperm of *S. ingentis,* these cells have been reported to possess depressed pH_is prior to activation. For example, the pH_i of unreacted *S. purpuratus* sperm is between 6.6 and 7.3, based upon measurements obtained with weak bases (Shackman *et al.,* 1981). Using 9-aminoacridine, the pH_i of the sperm of the starfish *Asterias amurensis* and *A. pectinifera* was reported to be 7.4–7.5 (Matsui *et al.,* 1986). In the hamster sperm, the intracrosomal pH has been measured to $\leq$5, also using 9-aminoacridine (Meizel and Deamer, 1978).

The ionophore nigericin exchanges K^+ or Na^+ for H^+ (the selectivity for K^+ over Na^+ is more than an order of magnitude), thus it is an electroneutral ionophore that dissipates proton gradients across cell membranes (Pressman, 1976; Johnson and Scarpa, 1976). As such, the pH_i of sperm in the presence of nigericin should more closely parallel the pH_0 of the ASW than in the low pH_0 experiments conducted without ionophore. The results of the nigericin induction experiments agree well with the measured pH_i changes that occur during the AR. Based on the DMO measurements, sperm decrease pH_i from 8.5 to between 7.8–8.0 as a result of the AR. Nigericin elicits filament formation at pH_0s 6.0–8.0 in sperm that have undergone exocytosis. Since the pH_0/pH_i gradient at pH_0s above 8.0 would not favor a nigericin induced pH_i decrease, filament formation would not be expected. Conversely, as the pH_0 is decreased, it would be expected that at some pH_0 an acid overload in the presence of nigericin would occur. This occurs between pH 5.75 and 6.0 in *S. ingentis* sperm.

Valinomycin, like low pH ASW and nigericin, does not elicit acrosomal exocytosis, but will induce acrosomal filament formation. However, unlike the other two, valinomycin is pH-independent over a wide pH_0 range (pH 5.75–8.0) at normal extracellular K^+ concentrations (10 m*M*). The ability of valinomycin to elicit filament formation does become sensitive to pH_0 at elevated extracellular K^+ concentrations. In 10 m*M* K^+ ASW filament formation proceeds at pH_0 6.0 and 8.0; no filaments are seen at pH_0 9.0. As the $[K^+]$ is increased to 40 m*M* (in 10 m*M* increments), filament formation declines approximately 50% at each incremental rise in $[K^+]$ in pH 8.0 ASW. At pH_0 9.0, filament formation is inhibited in $[K^+] \geq 10$ m*M;* however, filament formation will proceed if the K^+ concentration is below 10 m*M*. Valinomy-

cin transports only K^+ (the selectivity over Na^+ is greater than three orders of magnitude) across membranes and therefore is electrogenic (Johnson and Scarpa, 1976; Pressman, 1976). The results of the valinomycin/pH/K^+ experiments suggest that the ionophore is facilitating a K^+ efflux, however, they also suggest that the K^+ efflux does not in itself elicit filament formation. The fact that filament formation in pH 8.0 ASW is very sensitive to small changes in the extracellular K^+ concentration leads us to suggest that the pH_i decrease elicits acrosomal filament formation. The ionophore facilitates a K^+ efflux which results in an alteration of the sperm membrane potential (hyperpolarization?) and this change in membrane potential drives a proton influx.

This study has demonstrated that unactivated sperm of *S. ingentis* possess an unusually high resting pH_i, that they undergo a decrease in pH_i as a result of the AR, and that the pH_i decrease is associated with formation of the acrosomal filament. The pH_i measurements and shifts that occur during the AR in *S. ingentis* sperm must be viewed within the context of this unique system. These cells, after transfer to a female, are stored for several weeks or more in exoskeletal seminal receptacles during which time they undergo maturational and/or capacitational changes (Clark *et al.,* 1984). During storage they are separated from the seawater (pH *ca.* 8.0–8.2) by only the seminal plasm in which they are embedded. Thus, these cells probably maintain a pH_i in the same region as that found in their environment (seawater). At least two possibilities arise that would functionally explain why these sperm possess such a high unactivated pH_i: (1) the energetic costs of maintaining an elevated pH_i are less than if pH_i were depressed below physiological levels (*ca.* 7.0–8.0); or (2) since sperm undergo maturational/capacitational changes while in the seminal receptacles of the female, the elevated pH_i might be associated with these processes (*e.g.,* in the prevention of premature filament formation). These, of course, are not all inclusive nor are they mutually exclusive; rather, they are questions that await investigation.

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